

Report for 2005MN103B: Use of *Arthrobacter* *aureus* for Remediation of Groundwater Contaminated with Triazine Herbicides

Publications

- There are no reported publications resulting from this project.

Report Follows

Use of *Arthrobacter aurescens* for Remediation of Groundwater Contaminated with Triazine Herbicides

Principal Investigators:

Michael J. Sadowsky, Department of Soil, Water, and Climate, University of Minnesota
Lawrence P. Wackett, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota
Marc von Keitz, Biological Process Technology Institute, University of Minnesota

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Executive Summary:

The specific objectives of the proposed research are to evaluate the usefulness of the newly developed, stable and highly-active biocatalyst formulations for the bioremediation of atrazine-contaminated aquifer materials (water and sediments). The biocatalysts used in these studies were TrzN (triazine hydrolase) from *A. aurescens* strain TC1 and AtzB (hydroxyatrazine aminohydrolase) from *Pseudomonas* ADP. These two enzymes catalyze the first two steps in the atrazine biodegradation pathway, the dechlorination and subsequent hydrolysis of atrazine to non-toxic byproducts. In our studies we proposed to examine the stability, longevity, and activity of biocatalytic formulations in pilot scale simulated aquifers and wells. To achieve this goal, we firstly developed methods to overproduce the required enzymes, the used large scale fermentations to produce large quantities for cells for enzyme extraction. We are now developing methods to link these enzymes to solid support beads (EMPHAZE[®]) to maintain activity. This project is being done in conjunction with funding from the University of Minnesota Biocatalysis initiative, which provided funds to support the development of shelf-stable, highly active biocatalytic particles.

Methodology:

Expression and purification of TrzN. The TrzN was purified essentially as described by Shapir et al. (2006). Briefly, plasmid pAG, containing the chaperones groEL and groES, was transformed into *E. coli* BRL21(DE3) containing plasmid pET28b+::trzN. Strain BRL21(DE3) (pET28b+::trzN) was grown in LB medium containing kanamycin (50 µg/ml) and chloramphenicol (30 µg/ml) at 15°C, with shaking at 150 rpm. When cultures reached an optical density of 0.5 at 600 nm, the chaperones were induced by the addition of 0.0015% L-arabinose, and 1.5 µM IPTG (isopropyl-β-D-thiogalactopyranoside) was added after an additional 90 min of incubation at 15°C. Induced cells were grown for an additional 16 h under the same conditions, cultures were centrifuged at 10,000 x g for 10 min at 4°C, washed three times with 0.85% NaCl, and cell pellets were resuspended in 30 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 10% glycerol. Cells were broken by passage, three times, through a chilled French pressure cell operated at 140 Mpa, and cell-free extracts were obtained by centrifugation at 18,000 x g for 90 min at 4°C. Lysates were applied to a 5-ml HisTrap chelating column (Amersham Pharmacia Biotech, Piscataway, NJ), complexed with

Ni²⁺, according to the manufacturer's instructions. The column was washed with 15 ml 0.1 M sodium phosphate buffer, pH 7.0, followed by two washes with the same buffer supplemented with 0.1 M and 0.25 M imidazole, respectively. All buffers contained 10% glycerol. Enzyme was eluted from the column with 15 ml of 0.5 M imidazole in 0.1 M sodium phosphate buffer, pH 7.0, and the purified enzyme was concentrated using a Centricon-30 filtration unit (Amicon, Beverly, MA). Imidazole was removed from the enzyme preparation by dialysis, twice, at 4°C for 4 hr against 4 L of 0.1 M sodium phosphate buffer, pH 7.0 containing 10% glycerol. Enzyme purity and subunit molecular weight were estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay. Enzyme activity was measured by monitoring the disappearance of the substrate ametryn at 264 nm by using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Reactions (1 ml) were carried out at 37°C in 0.1 M sodium phosphate buffer, pH 8.0, containing 132 µM ametryn. Reactions were initiated by the addition of enzyme, and the molar absorbance at 264 nm for ametryn under these conditions was determined to be 5 mM⁻¹cm⁻¹. Enzyme activity was also followed using a colorimetric assay that we previously developed (Shapir et al. 2005).

Expression and purification of AtzB: The enzyme AtzB was essentially produced as described by Boundy-Milles et al. (1977). The *atzB* gene was cloned into plasmid pACYC184, under control of the *atzA* promoter. *E. coli*(pATZB) was grown in LB medium containing tetracycline (15 mg/ml) and ZnSO₄ incubated at 37°C. Overnight cultures were centrifuged at 12,000 x g for 10 min at 4°C, and pellets were washed twice with 25 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 6.9) and resuspended in the same buffer on ice. Cold cell suspensions were broken by three consecutive freeze-thaw cycles followed by sonication with a Biosonik sonicator (Bronwill Scientific, Rochester, N.Y.). Sonication was carried out three times at 80% probe intensity with intermittent cooling on ice. The broken cell suspensions were centrifuged at 17,000 x g for 90 min at 4°C to obtain crude cell extracts. Authentic samples of atrazine, hydroxyatrazine, and *N*-isopropylammelide were used to prepare 100-mg/ml stock solutions in 25 mM MOPS (pH 6.9). The crude extracts were diluted in 25 mM MOPS (pH 6.9) to obtain a final protein concentration of 50 mg/ml and amended with either atrazine, hydroxyatrazine, or *N*-isopropylammelide (100 mg/ml). Reaction mixtures were incubated at room temperature. We are currently optimizing large scale production and purification protocols for AtzB. Results of this work will be soon submitted to Applied and Environmental Microbiology.

Large scale fermentations: To produce sufficient quantities of the enzymes for immobilization and subsequent longevity studies, we firstly needed to produce large amounts of cells overproducing the enzymes. To do this, we used the fermentation facilities of the University of Minnesota Biotechnology Resource Center (BRC). A total of five fermentation runs were performed, totaling 750 liters of culture medium. This resulted in the production of 4,985 g of *E. coli* cells overproducing TrzN, and 10,340 g of cells overproducing AtzB. For fermentation of TrzN, *E. coli* BRL21(DE3) containing plasmid pET28b+::trzN and containing the chaperones groEL and groES was grown 300L DCI reactor at 15°C in TB medium containing kanamycin (50 µg/ml),

chloramphenicol (30 µg/ml), and ZnSO₄ (140 mg/l). When absorbance at 600nm reached 1.34, chaperone expression was induced by addition of L-arabinose to 0.015 g/L final concentration. After one doubling time (absorbance at 600nm = 2.56), trzN production was induced by the addition of IPTG to a final concentration of 2.0 uM. The culture was allowed to grow for 36 hours past the transition into stationary phase of growth for protein expression. 96 hr post-inoculation (final ABS₆₀₀ = 13), the culture was cooled and harvested by continuous centrifugation in the Sharples AS-16 centrifuge. The cell paste was frozen in liquid nitrogen and stored at -80°C.

Enzyme Activity of TrzN: Crude extract was tested for activity, and found to degrade 5.8 umoles atrazine/min/mg cells. This activity is quite excellent, as much smaller batch reactions (up to 2 L) produced a maximum degradation of 2.5 umoles atrazine/min/mg cells.

Project Significance:

TrzN catalyses the first reaction step in the degradation of as many as 400 different s-triazine compounds (Strong et al. 2002), chemicals that migrate into the environment as they are used in pesticide/herbicide/antimicrobial applications. This protein is being used in a prototype biocatalyst to reduce atrazine concentration below the maximum level allowed by law in drinking water of 3ppb. The Biocatalyst is formed by immobilizing enzyme onto solid beads (EMPHAZE™ beads manufactured by 3M, 140um diameter) via amino-functional ligands that form stable amide bonds with proteins. The resulting biocatalyst is packed into a plug-flow reactor configuration suitable for scale-up to purification of millions gallons drinking water per day.

Conservative analysis of research to date shows that reducing atrazine concentrations 93% (to 1ppb) in 1 million gallons per day would require a reactor containing 340 ft³ of media. Our current research focuses on improving this catalyst to reduce the required reactor size. The goal is to reduce capital and operating costs to confer affordability to governments in agricultural districts, where the need for this product is greatest.

Literature Cited:

Boundy-Mills, K. L., M. L. de Souza, L. P. Wackett, R. Mandelbaum, and M. J. Sadowsky. 1997. The atzB gene of *Pseudomonas* sp. strain ADP encodes hydroxyatrazine ethylaminohydrolase, the second step of a novel atrazine degradation pathway. *Applied Environ. Microbiol.* 63:916-923.

Shapir, N., C. Pedersen, O. Gil, L. Strong, J. Seffernick, M. J. Sadowsky, and L. P. Wackett. 2006. *Arthrobacter aurescens* TC1 is a Zinc Amidohydrolase. Submitted, *J. Bacteriology*.

Shapir, N., C. Rosendahl, G. Johnson, M. Andreina, M. J. Sadowsky, and L. P. Wackett. 2005. Substrate specificity and colorimetric assay for recombinant TrzN derived from *Arthrobacter aurescens* TC1. *Appl. Environ. Microbiol.* 71:2214-2220.

Strong, L. C., C. Rosendahl, G. Johnson, M. J. Sadowsky, and L. P. Wackett. 2002. *Arthrobacter aurescens* TC1 metabolizes diverse s-triazine ring compounds. *Appl. Environ. Microbiol.* 68:5973-5980.